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The Production of Vitamin B_{12}-Active Substances by Marine Bacteria

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ABSTRACT

The production of members of the vitamin-B_{12} family of compounds by 34 marine bacteria that were grown in a B_{12}-deficient medium was assayed. Using aliquots of the same bacterial culture, 70 per cent and 30 per cent had activity for the assay organisms *Escherichia coli* 113-3 and *Euglena gracilis*, *z* strain, respectively. Using the *Euglena* assay, no apparent relation was evident between the activities of the cell residues or corresponding supernatants and the relative amount of growth of each culture. However, of the 24 bacterial cultures tested, the supernatants of 42 per cent had activity, whereas the corresponding cell residues of 63 per cent had activity.

INTRODUCTION

Extensive reviews are available on members of the vitamin-B_{12} family of compounds dealing with their chemistry, functions in animal and microbial metabolism, distribution, production, and methods of measurement (McNutt 1952, Jukes *et al.* 1954, Hoff-Jørgensen 1954, and Ford and Hutner 1955). Burton and Lochhead (1951) and Lochhead and Burton (1955) have demonstrated that vitamin B_{12} is required by certain soil bacteria. Vitamin B_{12}-active compounds have been shown to be essential or stimulatory for the growth of various marine microorganisms (Hutner and Provasoli 1953, Droop 1954, Lewin 1954, Sweeney 1954, Droop 1955, and Wilson and Collier 1955). Such observations suggest that vitamin B_{12}-active compounds may have a vital role in the economy of the oceans, in production of algal blooms, and in population successions.

In previous studies (Starr 1956), the vitamin-B_{12} content of the suspended matter of estuarine and oceanic waters was assayed. The present investigation was undertaken to learn more about the vitamin-B_{12} cycle in marine environments. Information gained from this and related studies encompassing the vitamin cycles and the roles of organic compounds of the oceans may add materially to our understanding of marine ecology and its application to marine fisheries.

In this investigation microbiological assay procedures were used to measure the production of vitamin B_{12}-active compounds by 34 different bacteria isolated from marine environments. Relative levels of production were compared using two different microbiological assay organisms having different spectra of activity for members of the vitamin-B_{12} family of compounds.

EXPERIMENTAL

The 34 bacteria screened for production of vitamin B_{12}-active substances represent different morphological and physiological groups that are maintained in our permanent stock culture collection. They have not as yet been identified. They were isolated from a variety of marine samples by techniques described in a previous publication (Starr and Jones 1957).

The bacteria were grown in a vitamin B_{12}-deficient medium of the following composition: NaCl, 0.3 g; $MgSO_4 \cdot 7H_2O$, 0.02 g; KNO_3, 0.02 g; NH_4Cl, 0.01 g; K_2HPO_4, 0.04 g; Na acetate, 0.01 g; sucrose, 0.1 g; glycine, 0.01 g; yeast extract 0.02 g; and distilled water to 100 ml. The pH was adjusted to 7.5–7.7 with 1N NaOH. After 10 days' incubation at 26°C ± 2°C, aliquots of each culture were diluted 1:10 with a buffer at pH 4.5. The buffer contained 8.2 g/L of KH_2PO_4 and 0.5 g/L of $Na_2S_2O_5$. The diluted cultures were hydrolyzed in a boiling water bath for 20 min. After cooling, a second 1:10 dilution was made from the first with the same buffer. These dilutions, 1:10 and 1:100, of each culture were diluted

further with distilled water to give the following final assay dilutions: 1:10, 1:20, 1:50, 1:100, 1:200, 1:500, 1:1000.

The following procedures were used in order to determine if vitamin B_{12}-active substances were confined to the bacterial cells or released into the medium. Twenty-four of the 34 bacteria were grown in 400 ml of medium in 1000 ml Erlenmeyer flasks. The cultures were incubated at 26° ± 2°C on a rotary shaker for 6 to 8 days. Twenty-ml aliquots of each culture were centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was decanted, and final dilutions were prepared from it. The cell residues were suspended in 10 ml of the phosphate-metabisulfite buffer, and final dilutions were made. Other aliquots of each culture were used for turbidimetric growth measurements using a Klett-Summerson colorimeter and for pH measurements.

Samples were assayed for vitamin-B_{12} activity using *Euglena gracilis*, *z* strain, (Pringsheim's) which was obtained through the courtesy of Dr. L. Provasoli. It is now available from the American Type Culture Collection. The methods and procedures were essentially those of Hutner *et al.* (1956) with minor modifications. Stock cultures of *Euglena* were maintained in screw-cap tubes containing: K_2HPO_4, 2 mg; $Na_2 \cdot$ citrate$\cdot 2H_2O$, 2 mg; $FeCl_3 \cdot 6H_2O$, 0.2 mg; peptone, 60 mg; trypticase, 16 mg; yeast extract, 5 mg; and made to volume with 100 ml of distilled water. The pH was adjusted to 6.5. Assays were performed in screw-cap tubes (size 125 x 20 mm) using the basal medium, "dry mix", described by Hutner *et al.* (1956). Two and one-half ml of the double-strength basal medium were added to 2.5 ml of each dilution of the sample. Duplicate standard curves were made using commercial vitamin B_{12} to give the following final concentrations (mμg/100 ml): 0.0, 0.05, 0.12, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, and 1.0. All media were autoclaved at 15 lb for 10 min prior to inoculation. A 6 to 8 day-old *Euglena* culture grown in the stock culture maintenance medium was used for inocula. A bacteriological loop was used to transfer approximately 0.05 ml to 12 ml of the basal medium. One drop of the resulting suspension (approximately 0.08 ml) was used as inoculum per assay tube. Although it has been shown (Østergaard Kristensen 1954) that the supernatant fluid of well-grown *Euglena* cultures contains material which inhibits the growth of *Euglena* in concentrations as low as 1 in 2000 (Hutner *et al.* 1956), our dilution represented a safe margin with a concentration of approximately 1:15,000. Cultures were incubated under a battery of fluorescent lights at 27° ± 1°C for 6 days. Growth was measured using a Klett-Summerson colorimeter.

The other assay organism was the mutant strain *Escherichia coli* 113-3 obtained through the courtesy of Dr. B. D. Davis. The methods of assay were essentially those described by Burkholder (1951) and modified as described previously (Starr 1956). Stock cultures of *E. coli* were maintained on nutrient agar slants and were transferred weekly. Assays were performed as described for *Euglena*. Standard curves were made in duplicate to give the following final concentrations of commercial vitamin B_{12} (mμg/100 ml): 0.0, 1.0, 2.0, 5.0, 10.0, 15.0, 20.0, 30.0, and 40.0. All media were autoclaved at 10 lb for 3 min prior to inoculation. Inocula were prepared from a 24-hr slant of *E. coli* by carefully transferring a small amount of growth with a fine platinum needle to 12 ml of single strength basal medium. One drop (approximately 0.08 ml) of this cell suspension was used per tube. Experiments were incubated for 18 to 20 hr at 30°C on a tissue-culture apparatus rotating 20 to 24 rpm. Growth was measured turbidimetrically with a Klett-Summerson colorimeter.

RESULTS

Typical assay response curves for *Euglena* and *E. coli* to increasing concentrations of commercial vitamin B_{12} are shown in Figure 1.

The results of the preliminary screen to determine the numbers of bacteria producing vitamin B_{12}-active substances are given in Table 1. Using aliquots of the same culture, 70 per cent and 30 per cent of the isolates

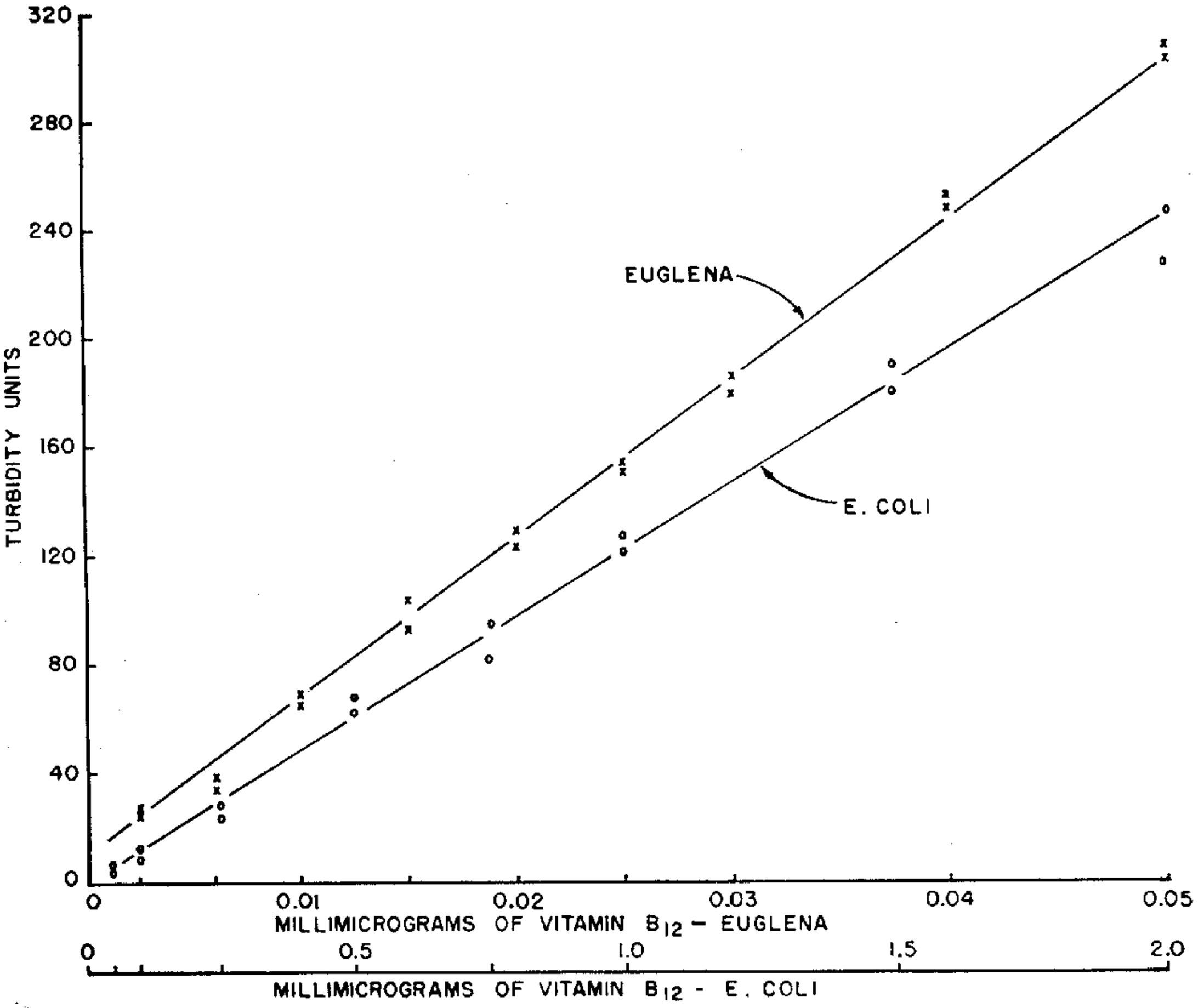

FIG. 1. Responses of *Euglena gracilis*, *z* strain, and *Escherichia coli* 113-3 to increasing concentrations of vitamin B_{12} per 5 ml of medium.

showed activity by the *E. coli* and *Euglena* assays, respectively. In no instance was a culture positive for *Euglena* and negative for *E. coli*.

The relative levels of vitamin B_{12}-activity produced by the different cultures ranged from 0 to 5.0 mμg/ml as measured by the *Euglena* assay and from 0 to 18.4 mμg/ml as measured by the *E. coli* assay.

Other experiments were designed to determine if vitamin-B_{12} activity was confined to the bacterial cells or released into the medium. The cell residues and supernatants of mass cultures were obtained and processed as described. Aliquots of them were assayed with *Euglena*. For comparison, the same cultures used in the previous experiments (Table 1) were screened.

Of the 24 cultures tested, the cell residues of 63 per cent showed activity, whereas the corresponding supernatants of only 42 per cent showed activity (Table 2).

There was no apparent relationship between the amount of growth (turbidity readings) or final pH of each culture and the levels of vitamin-B_{12} activity of either the cell residues or supernatants. For example, the vitamin-B_{12} activity of culture 266 which grew meagerly (turbidity, 15) was confined to the cell residue. Other cultures (126 and 222B) which grew prolifically produced relatively less activity. In no instance did a supernatant have activity and its corresponding cell residue have none, although the reverse situation occurred a number of times.

TABLE 1. *Comparative responses of* Escherichia coli *113-3 and* Euglena gracilis, z *strain, to aliquots of the same bacterial cultures*

Units of activity in terms of mμg vitamin B_{12} per ml.

Culture No.	*E. coli*	*Euglena*
Control	0	0
112	1.2	0.50
116	3.4	1.36
117	3.8	1.38
121	13.0	4.20
122	0	0
126	1.2	0
127A	0	0
127B	0	0
143	1.1	0
144	0.70	0
148	0	0
149	0.98	0
202	1.4	0
203	0	0
205	2.8	1.36
209	0.75	0.24
215	0.70	0
222A	0	0
222B	1.3	0
233	5.0	1.90
238	1.0	0
245	16.7	4.30
249	1.3	0
250	0	0
255	0	0
259	1.1	0
260	0.40	0
266	1.4	0
276	1.4	0.60
279	0.95	0
286	0	0
287	0	0
288	3.4	0.36
306	18.4	5.00

TABLE 2. *Response of* Euglena gracilis, z *strain, to preparations of the supernatants and cell residues of bacterial cultures, and accompanying data on pH and Klett units of growth*

Units of assay activity in terms of mμg vitamin B_{12} per ml.

Culture No.	Klett units	Final pH	Cell residues	Supernatants
Control	0	7.4	0	0
112	107	7.0	7.00	0.048
116	64	8.4	4.50	0.200
117	66	8.2	2.68	0.035
121	35	7.4	16.2	0.550
122	48	8.4	0	0
126	295	6.3	0.16	0
127A	45	8.2	0.02	0
127B	45	8.2	0	0
143	289	6.4	0	0
144	54	6.9	0	0
148	40	5.9	0	0
203	20	8.2	0	0
205	168	6.6	13.3	0.059
209	110	7.3	1.50	0.004
222A	29	8.3	0	0
222B	222	5.8	0.05	0
233	65	6.8	1.88	0.038
238	107	8.0	0	0
249	259	6.7	0.10	0
250	29	8.5	0	0
266	15	7.7	11.9	0
276	215	7.7	14.0	0.055
279	182	5.0	0.21	0.022
306	30	7.5	3.84	0.870

DISCUSSION

The responses of *E. coli* and *Euglena* to members of the naturally occurring B_{12}-vitamins are discussed in detail by Coates and Ford (1955), Ford and Hutner (1955), and Hutner *et al.* (1956). *E. coli* responds to non-specific substances such as methionine and to Factor B, whereas *Euglena* does not respond to either of them. Factor B (Kon 1955) is the non-nucleotide moiety common to several of the B_{12}-vitamins. If this factor or any one of a number of nucleotides are present, some organisms can complete the synthesis of a form of "vitamin B_{12}" containing that particular nucleotide (Coates and Ford 1955). From the standpoint of the requirements of man, the clinically active vitamin B_{12} or cyanocobalamin is of particular importance. From the standpoint of the growth requirements of marine microorganisms, the pseudovitamin B_{12} and related factors may prove to be as important as cyanocobalamin. Various investigators have demonstrated that cyanocobalamin is a growth factor required by some diatoms and flagellates (Provasoli and Pintner 1953). In addition, Droop (1955) showed that the cyanocobalamin required by the pelagic diatom *Skeletonema costatum* was spared by pseudovitamin B_{12}, Factor A, and Factor B. His noteworthy observations on the lack of specificity of the form of vitamin B_{12} preferred by this diatom indicates the probable importance of "cobalamins" (members of the vitamin-B_{12} family of compounds

in addition to cyanocobalamin) in the economy of the sea.

In this investigation vitamin B_{12}-activity produced by marine bacteria was measured with *Euglena* and *E. coli* both of which have different spectra of activity. Of the 34 cultures screened, 30 per cent and 70 per cent had activity for *Euglena* and *E. coli*, respectively. These data are in agreement with those of Ericson and Lewis (1953) who screened 34 cultures using the *E. coli* 113-3 cup-plate assay and found that 70 per cent were producers. Burton and Lochhead (1951) found that 65 per cent of their bacterial cultures were producers. They examined terrestrial isolates using the *Lactobacillus lactis* assay. A table describing the responses of the different assay organisms to vitamin B_{12}-active compounds is given in Ford and Hutner (1955).

In all cases involving aliquots of the same culture which were tested with both assay organisms, the *E. coli* assay gave decidedly higher values (Table 1). This observation indicates the broader spectrum of response of *E. coli* to vitamin B_{12}-active substances compared to *Euglena* and the wide variation of specific vitamin-B_{12} activity produced by different cultures under similar conditions. The maximum levels of vitamin-B_{12} activity produced by culture no. 306 were 5.00 and 18.4 mμg/ml for *Euglena* and *E. coli*, respectively. Using the *L. lactis* assay Burton and Lochhead (1951) found terrestrial bacteria which produced over 500 mμg activity per ml of culture medium. Burkholder and Burkholder (1956) found marine bacteria which produced up to 150 mμg/ml. They used the *E. coli* agar plate method. Using the *E. coli* 113-3 cup-plate assay, Ericson and Lewis (1953) found marine bacteria which produced 0.005–140 mμg/ml. They stated that a level of approximately 10 mμg/ml is more comparable to those obtained for bacteria isolated from seawater and seaweed. This figure is in agreement with the levels (0.40–18.4 mμg/ml by *E. coli*) found during this investigation.

In experiments designed to determine if vitamin B_{12}-activity was confined to the cell residues or released into the medium, results varied with each culture as shown in Table 2. In general intracellular levels of vitamin B_{12} were higher than extracellular. No apparent relationship was evident between the levels of activity of either the cell residues or supernatants and the final bacterial growth or pH of the medium.

As shown in this and related studies by other investigators, the amount and specificity of B_{12}-activity produced by a particular bacterial culture varied with the conditions of the experiment and methods of measurement.

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